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Analysis of promoter activity of cotton lipid transfer protein gene *LTP6* in transgenic tobacco plants

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Abstract

A cotton (*Gossypium hirsutum*) genomic clone (1.7-kb DNA insert) harboring the lipid transfer gene *Ltp6* specifically expressed in fiber cells had been previously isolated and characterized. By using PCR amplification, the 447 bp *Ltp6* promoter and a series of 5′ deletions of the promoter were generated and cloned into a pBI101 plasmid upstream of the GUS (β-glucuronidase) reporter gene. These constructs were introduced into *Agrobacterium tumefaciens* LBA4404, and leaf disks of tobacco (*Nicotiana tabacum* L.) were transformed with *A. tumefaciens* LBA4404 carrying various promoter-GUS pBI101 plasmids. Histochemical analyses of the transgenic tobacco seedlings indicated that the *Ltp6* promoter (from nt −447 to −1, undeleted) directed GUS expression only in trichomes (hair cells). Fluorometric GUS assays showed that the promoter activity of the undeleted *Ltp6* promoter was at least 1000 times weaker than that of the 35S promoter of cauliflower mosaic virus (CaMV). Sequential deletions of the promoter gradually decreased the expression level of the GUS gene. No GUS activity was observed when the 5′ deletion of the *Ltp6* promoter reached to nt −86, which removed the putative CAAT and TATA promoter elements.

Keywords: *Agrobacterium*-mediated transformation; β-Glucuronidase; Histochemical GUS assay; Fluorometric GUS assay; Polymerase chain reaction; Trichomes

1. Introduction

Cotton fibers are differentiated epidermal cells developed from the ovule. Cloning of fiber genes and the study of their expression and regulation will be the first step in our understanding of the molecular mechanism of fiber development. We have recently isolated and characterized several lipid transfer protein genes (*Ltp3*, *Ltp6*, and *Ltp12*) specifically expressed in fiber cells [1,2]. They are highly expressed during the elongation phase of fiber development and are developmentally regulated. The encoded fiber lipid transfer proteins (LTPs) have the general characteristics of plant nonspecific LTPs (nsLTPs). Several physiological functions of plant nsLTPs have been proposed: (1) participation in cutin synthesis [3,4]; (2) response to environmental stresses [5,6]; and (3) defense against bacterial and fungal pathogens [7–10]. Based on their expression patterns and the presence of a signal peptide in the N-terminal of nucleotide-derived LTP protein sequences, we have proposed that the cotton LTPs may participate in cutin synthesis during fiber development [1].

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The cotton *Ltp6* gene, isolated from a λ gt10 genomic library, is located within a 1.7-kb EcoRI DNA insert and 5′ flanked by 447 bp relative to its translation initiation site [2]. Since the expression of *Ltp6* is fiber-specific and developmentally regulated, the 5′ upstream sequences are likely to contain regulatory elements conferring spatial and temporal controls. In this study, the 5′ flanking region (from nt −447 to −1) and a series of deletions of this region were tested for promoter activity in transgenic tobacco plants. When fused with the GUS gene the 447 bp 5′ flanking region of the *Ltp6* gene showed tissue-specific GUS expression in transgenic tobacco plants. GUS activity was found only in trichomes (hair cells) of young tobacco leaves. The results showed that cotton fibers and tobacco trichomes, both belonging to differential epidermal cells, may share common regulatory elements for tissue-specific expression.

2. Materials and methods

2.1. Construction of promoter-GUS fusions

The 447 bp *Ltp6* promoter and a series of 5′ deletions of this promoter were constructed by PCR amplification [11]. A total of six primers were designed for PCR, and their nucleotide sequences were as follows: GP1, 5′-CGAAGCTTGAATTC-CCCTTCTGTTTTAG-3′ (from nt −447 to −428); GP2, 5′-GCCATGGATCCGATTAATTA-CAAG-3′ (from nt −16 to −1); GP5, 5′- CC-GAAGCTTCATCTATTTGACTCG-3′ (from nt −274 to −258); GP6, 5′-CCGAAGCTTGGCA-TTGAATTAGGGCA-3′ (from nt −198 to −180); GP7, 5′-CCGAAGCTTCCATATTCCTT-CACTCA-3′ (from nt −122 to −104); GP8, 5′-CAAAAGCTTCCCTCCAACCAGCAATC-3′ (from nt −86 to −70). GP2 was utilized as a 3′ primer, and the other five oligonucleotides were used as 5′ primers. A *Hin*dIII restriction enzyme site (AAGCTT) was created at the 5′ end of the 5′ primers (GP1, GP5, GP6, GP7, and GP8), and a *Bam*HI site (GGATCC) was generated at the 5′ end of the GP2 primer. PCR was performed for 30 cycles (94°C for 1 min, 55°C for 1 min, and 72°C for 2 min) using *Taq* DNA polymerase and recombinant M13mp19 containing the *Ltp6* gene insert as the template. The promoter PCR products were digested with *Bam*HI and *Hin*dIII and individually cloned into the *Bam*HI and *Hin*dIII sites of a promoterless binary plasmid pBI101 (Clontech) which contains a 1.87 kb GUS cassette. The 5′ flanking region of the *Ltp6* gene and six primers used for PCR are shown in Fig. 1, and the five promoter-GUS constructs were designated as pBI101-*Ltp6*-447, pBI101-*Ltp6*-274, pBI101-*Ltp6*-198, pBI101-*Ltp6*-122, and pBI101-*Ltp6*-86 (Fig. 2).

Fig. 1. The 5′ flanking region of the *Ltp6* gene and six specific primers for PCR amplification. The initiation codon ATG and CAAT and TATA boxes are double-underlined. The direction and nucleotide sequences of the six primers are indicated. The A nucleotide of the initiation codon ATG is numbered as +1.

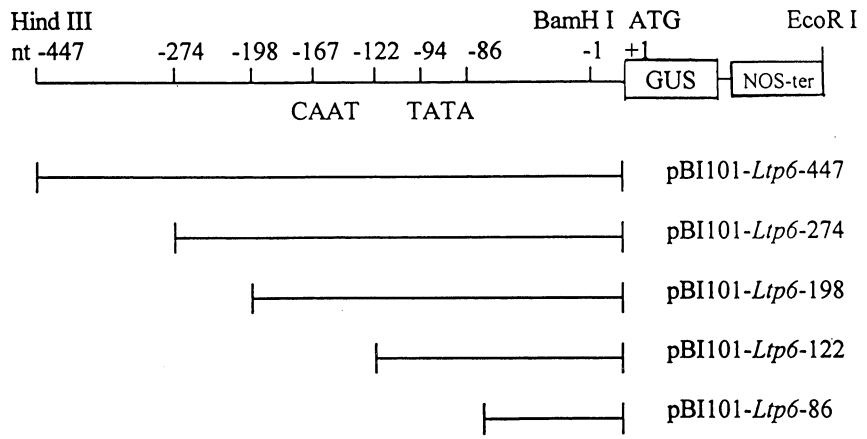

Fig. 2. Schematic diagram of *Ltp6* promoter-GUS constructs. The undeleted and 5′ deleted *Ltp6* promoter fragments were generated by PCR amplification and fused with the GUS gene in pBI101. NOS-ter indicates the terminator of the nopaline synthase gene.

2.2. Production of transgenic tobacco plants

The five recombinant pBI plasmids were individually introduced into *Agrobacterium tumefaciens* LBA4404 cells by using a freeze–thaw method [12]. Plasmids were isolated from transformed *Agrobacterium* colonies [12] and the promoter construct was confirmed by PCR amplification with specific primers of the *Ltp6* promoter. The transformed *Agrobacterium* strain LBA4404 was used to transform tobacco (*Nicotiana tabacum* L.) with the standard leaf disk method [13]. Transgenic tobacco plants were cultivated in a growth chamber (Percival, model I-35) at 28°C with 16-h illumination per day.

2.3. Histochemical GUS assay

Young shoots or leaves of nontransformed and transgenic tobacco plants were cut out and stained in 0.1% X-Gluc solution as described by Jefferson [14]. After incubation at 37°C overnight, the chlorophyll pigments were removed by consecutive extractions with 50, 70, and 100% ethanol. The tissue sections were then kept in 0.1 M sodium phosphate buffer, pH 7.0, and examined under a stereo microscope (Olympus, model SZH10).

2.4. Fluorometric GUS activity assay

At least five individual transgenic seedlings from each *Ltp6* promoter-GUS construct were used for the fluorometric assay. For nontransformed tobacco plants and transformed plants containing the CaMV 35S promoter only two individual plants were utilized for the assay. Leaf tissues of tobacco plants were ground with liquid nitrogen and homogenized in GUS extraction buffer containing 0.1 M phosphate buffer, pH 7.0, 2 mM Na_2EDTA, 2 mM DTT, and 5% glycerol. GUS activity in the supernatant fraction was assayed by measuring the fluorescence of the reaction product 4-methylumbelliferone (4-MU) formed by the GUS-catalyzed hydrolysis of the substrate 4-methylumbelliferyl-β-D-glucuronide (4-MUG) as described by Jefferson [14]. Fluoresence was measured with a Hoefer TKO 100 fluorometer with a fixed excitation wavelength at 365 nm and an emission wavelength at 455 nm. The protein concentration of the GUS extracts was determined using the Bradford method [15].

2.5. Genomic reconstruction

The copy number of *Ltp6*-GUS integrated into the tobacco genome was determined by genomic reconstruction. Based on the haploid genomic content (3.2×10^{-12} g) and size (2.9×10^{9} nt) of the tobacco plant, 44 pg of pBI101-*Ltp6*-447 (12.65 kb) per 10 μg of nuclear tobacco DNA are equal to one gene copy equivalent. Therefore, 44 pg (one gene copy), 88 pg (two gene copies), and 220 pg (five gene copies) of the pBI101-*Ltp6*-447 plasmid were each mixed with 10 μg of wild type tobacco genomic DNA and then digested with restriction enzymes *Hin*dIII and *Eco*RI (10 U each) in a reaction buffer containing 10 mM Tris, pH 7.8, 10 mM $MgCl_2$, 50 mM NaCl, 1 mM 2-mercap-

toethanol at 37°C. In addition, 10 μg of tobacco genomic DNA from nontransformed plants and six individual transgenic plants carrying the *Ltp6*-447-GUS construct were also digested with the two enzymes under the same conditions. The *Hin*dIII and *Eco*RI digested DNA samples were electrophoresed on a 1% agarose gel, transferred to a nylon membrane, and hybridized with a 2.3 kb *Hin*dIII–*Eco*RI fragment (*Ltp6*-GUS fusion) and ^{32}P-labeled by the random priming labeling method [16].

3. Results

3.1. Undeleted Ltp6 promoter directed GUS expression in trichomes of transgenic tobacco plants and progressive 5′ deletion of promoter altered tissue-specific expression

The 447 bp *Ltp6* promoter region was progressively deleted from the 5′ end to generate five truncated promoters fused with the GUS gene. The leaves from tobacco seedlings transformed with pBI121 (CaMV 35S promoter) and the five pBI101-*Ltp6* deletion constructs and nontransformed plants were used for the histochemical GUS assay. GUS expression was observed in leaf tissues from transgenic tobacco plants containing *Ltp6*-447-GUS (Fig. 3A), *Ltp6*-274-GUS (Fig. 3B), and *Ltp6*-198-GUS (Fig. 3C). When examined under a stereo microscope, the blue color representing GUS activity was found only in hair cells (trichomes) of leaf tissues carrying the *Ltp6*-447-GUS (Fig. 3A), but was present in trichomes and guard cells in the *Ltp6*-274-GUS (Fig. 3B) and *Ltp6*-198-GUS (Fig. 3C) transgenic plants. In the positive control with plants transformed with pBI121 containing the 35S promoter, the blue color was extensive in all areas including the trichome cells (Fig. 3D), which indicated nonspecific tissue expression. When the deletions of the *Ltp6* promoter past the CAAT (pBI101-*Ltp6*-122) and TATA (pBI101-*Ltp6*- 86) boxes were used for transformation, GUS activity could not be detected in the leaf tissue (Fig. 3E and 3F). The trichomes were colorless and transparent and were the same as those of nontransformed tobacco plants (Fig. 3G).

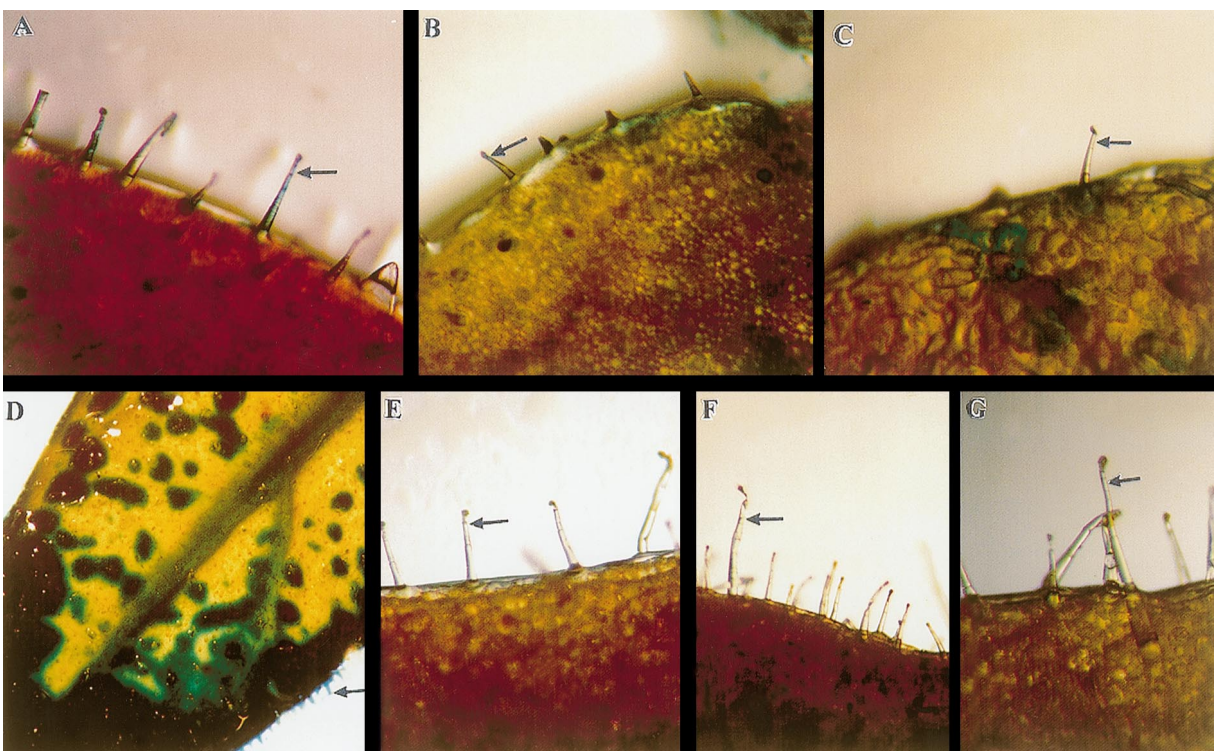

Fig. 3. Histochemical analysis of wild type tobacco leaf tissues (G) and transgenic tobacco seedlings containing the construct of: (A) *Ltp6*-447-GUS, (B) *Ltp6*-274-GUS, (C) *Ltp6*-198-GUS, (D) CaMV 35S promoter-GUS, (E) *Ltp6*-122-GUS, and (F) *Ltp6*-86-GUS. The arrow indicates a trichome.

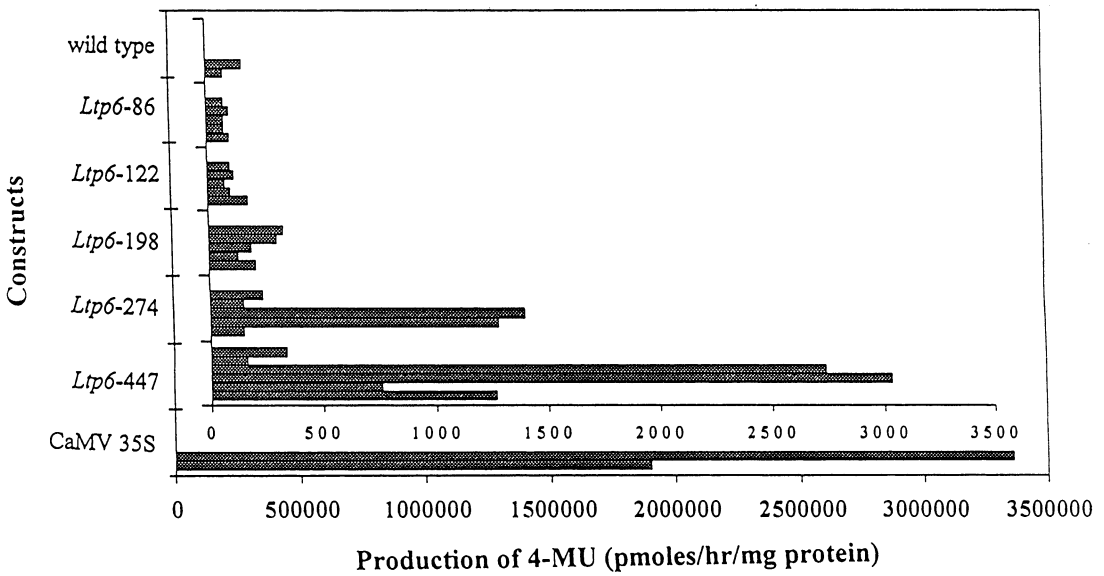

Fig. 4. Fluorometric GUS assay in transgenic tobacco plants carrying various *Ltp6* promoter- GUS constructs. The six transgenic plants 1–6 harboring the *Ltp6*-447-GUS are shown from the bottom to the top. The GUS activities are expressed as pmol 4-MU/mg × h of extracted protein, and each bar represents the average value of two duplicate measurements.

3.2. Progressive 5′ deletion of Ltp6 promoter decreased promoter activity

Quantitative fluorometric GUS assays in transgenic tobacco plants with different *Ltp6* promoter-GUS constructs are shown in Fig. 4. All tobacco plants transformed with pBI101-*Ltp6*-447, pBI101-*Ltp6*-274, and pBI101-*Ltp6*-198 constructs contained GUS activity, but the activity gradually decreased as the *Ltp6* promoter was progressively deleted. The average GUS activity of *Ltp6*-447-GUS transgenic plants was about twice that of *Ltp6*-198-GUS transgenic tobacco plants. However, when the deletion reached to nt −122 (post-CAAT box) and nt −86 (post-TATA box), no GUS activity was found in transgenic tobacco plants containing *Ltp6*-122-GUS and *Ltp6*-86-GUS constructs. Compared with pBI121 transformed tobacco plants which carry the CaMV 35S promoter, the average GUS activity of *Ltp6*-447-GUS (undeleted) transgenic tobacco plants was at least 1000 times weaker than that of the CaMV 35S promoter. The results of the histochemical GUS assay were consistent with the data from the fluorometric GUS assay.

3.3. Copy numbers of the Ltp6-GUS gene were not correlated with levels of GUS expression

Southern analysis indicated that a 2.3 kb DNA band, which represented the *Ltp6* promoter (447 bp) fused with the GUS gene (1.87 kb), hybridized to the probe in all transgenic tobacco plants and pBI101-*Ltp6*-447 plasmids, but was absent in nontransformed tobacco plants (wild type) (Fig. 5). Comparisons of the intensity of hybridized bands from transgenic plants carrying the *Ltp6*-447 construct with that of copy number constructions indicated the following: the transformant line 1 contained two copies of the *Ltp6*-GUS gene; lines 2, 3, and 6 had five copies of the gene; line 4 contained three or four copies; and line 5 had only one copy. Results from Fig. 4 were used to correlate the possible relationship between copy numbers of the *Ltp6*-GUS gene and the levels of gene expression. Three *Ltp6*-447 transformant lines, 2, 3, and 6, contained the same copy number (five) of the gene; however, the GUS expression in line 3 was the strongest (3040 pmol 4-MU/mg × h), with line 2 being the next (760 pmol 4-MU/mg × h) and line 6 the lowest (340 pmol 4-MU/mg × h). Further, while transformant line 1 contained two gene copies, its GUS gene expression was much higher (1270 pmol 4-MU/mg × h) than lines 2 and 6. These results indicated that there was no obvious relationship between copy numbers of the gene and the intensity of gene expression. The difference in the expression of the *Ltp6*-GUS gene from different transformant lines might be influenced by its insertion into various sites of either 'active' or 'inactive' chromatin-a positional effect.

4. Discussion

In this study, we have found that the 447 bp 5′ flanking region of the *Ltp6* gene, when fused with the GUS gene (pBI101-*Ltp6*-447), directed tissue-specific GUS expression in transgenic tobacco plants. GUS activity was only found in trichomes (hair cells) of young leaves. When the 5′ deletion reached to nt −274 (pBI101-*Ltp6*-274), GUS activity was detected in the trichomes and guard cells. The promoter regions of several plant *Ltp* genes have also been analyzed by the GUS method [4,17,18]. The 972 bp in the 5′ flanking region of a broccoli (*Brassica oleracea*) *Ltp* gene (*wax9D*) was also shown to direct GUS expression in transgenic tobacco plants in the epidermis of leaves, stems and flower petals, sepals, ovules, and trichomes [4]. The 1149 bp 5′ flanking region of the *Arabidopsis Ltp1* gene also directed GUS expression in epidermal cells of various tissues of transformed *Arabidopsis* [17]. When the progressive 5′ deletions past the CAAT and TATA boxes (pBI101-*Ltp6*-122 and pBI101-*Ltp6*-86 constructs), no GUS activity was detected in transgenic tobacco plants, which confirmed that these two boxes with conserved sequences served as basal elements for the transcription. Since cotton fibers and tobacco trichomes are both differentiated epidermal cells, they may share common regulatory elements for tissue-specific expression. The GUS expression patterns from the pBI101-*Ltp6*-447 and pBI101-*Ltp6*-274 constructs strongly suggested that the nt sequence from −447 to −274 may contain fiber regulatory elements. The use of the GUS reporter gene to detect tissue-specific expression can generate artifacts. Different reporter genes and in situ mRNA hybridization analysis are necessarily used to confirm the GUS results.

The promoter function of two cotton fiber genes, *E6* and *FbL2A*, was analyzed in transgenic cotton plants [19,20]. The 2.5 kb upstream sequences of *E6* was able to direct the expression of a carrot extensin in cotton fiber cells [19]. The 2.3 kb 5′ flanking region of *FbL2A*, was shown to direct the expression of bacterial acetoacetyl-coenzyme A reductase and poly-hydroxyalkanoic acid synthase in transgenic cotton in a fiber specific and developmentally regulated manner [20]. To determine whether the *Ltp6* promoter will direct the fiber-specific GUS expression, transgenic cotton plants carrying the *Ltp6*-GUS construct will have to be generated. The *Agrobacterium*-mediated transformation and the particle bombardment of meristematic tissues can be used to generate transgenic cotton plants. At present, developing transgenic cotton plants to dissect fiber promoters takes a long time and is also labor intensive and expensive. How well the trichome expression in tobacco leaves represents the expression requirements in cotton fibers remains to be determined.

The transcriptional regulation of gene expression in plants can be mediated by interaction between sequence-specific transcription factors and their target regulatory elements generally located at the 5′ upstream regions. Besides the two basal promoter elements, TATA and CAAT boxes, additional *cis* elements such as the C-box (nnGACGTCnn) [21], G-box (CACGTG) ele-

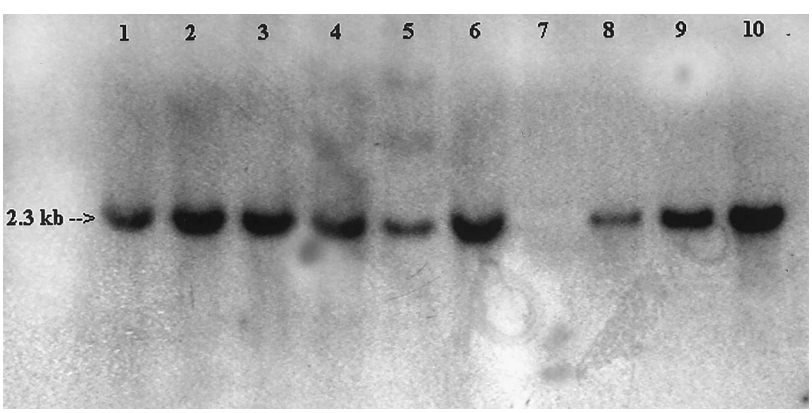

Fig. 5. Genomic reconstruction analysis of the *Ltp6*-GUS insert in transgenic tobacco plants. The 2.3 kb *Ltp6*-GUS fragment is used as a hybridization probe. Lanes 1–6 indicate six individual transgenic tobacco plants containing the *Ltp6*-447-GUS construct. Lane 7 is the nontransformed tobacco plant, and lanes 8, 9, and 10 represent 1, 2, and 5 copies of the *Ltp6*-GUS insert per haploid genome equivalent.

ments [22] and the Hex motif (TGACGTGGC, a hybrid of C and G box elements), play key roles in plant promoters to modulate the signal pathways for developmental and environmental regulation. Putative ABA-responsive elements (ABRE) containing the ACGT central core sequence have been found in the rice *Ltp1* and *Ltp2* genes [18] and tomato (*Lycopersicon pennellii*) *LpLtp1* and *LpLtp2* genes [23]. A putative ACGT-containing promoter element (G-box-like element) is located from nt −278 to −275 in the 5′ flanking region of the *Ltp6* gene. It will be interesting to find out whether the fiber transcription factors can recognize this ACGT core sequence. The trichome initiation in *Arabidopsis* requires the expression of the *Transparent Testa Glabra* (*TTG*) and *Glabrous1* (*GL1*) genes [24]. The TTG gene encodes a protein that regulates the expression of a *myc*-like transcription factor [25], and the *GL1* gene encodes a *myb*-type transcription factor [26]. Longuercio et al. have recently cloned six cotton *myb* genes and found the expression of some of them was correlated with developmental stages in cotton fiber development (manuscript in preparation). Four putative recognition sites (consensus sequence TGGTTAG) for MYB-related proteins are found to be located from nt −202 to −196, −243 to −237, −302 to −308, and −339 to −332 in the *Ltp6* promoter. The cotton MYB protein can be over-expressed in *Escherichia. coli* and used to determine whether it binds to these putative *Ltp6* MYB-binding sequences by the electrophoretic mobility shift assay (EMSA) and the DNaseI footprinting technique [27].

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